"Studies on Some Aspects of Carbohydrate Metabolism in Tissue Schizont Stages (Exo-erythrocytic Forms) of Malarial Parasite."

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BY
SHAHID UMAR
M. Sc.

BIOCHEMISTRY DIVISION
CENTRAL DRUG RESEARCH INSTITUTE
CHATTAR MANZIL PALACE
LUCKNOW 226 001

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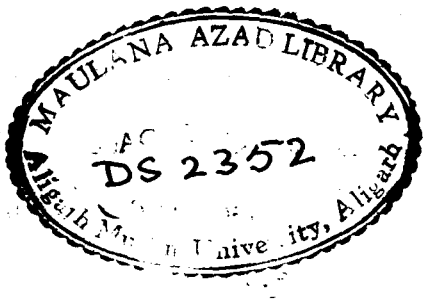

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DS2352

Telex : 0535-286
Telegram : CENDRUG
Phone : 32411-18 PABX

केन्द्रीय औषधि अनुसंधान संस्थान
छत्तर मंजिल, पोस्ट बाक्स नं० 173
लखनऊ 226 001 (भारत)
CENTRAL DRUG RESEARCH INSTITUTE
Chattar Manzil, Post Box No. 173
LUCKNOW-226001 (INDIA)

No. Biochem./MKS/T&E/89.

Date 8.12.89

DR. M.K. SAHIB
Asstt. Director
Div. of Biochemistry

CERTIFICATE

This is to certify that the work embodied in this thesis entitled "Studies on some aspects of carbohydrate metabolism in tissue schizont stages (exo-erythrocytic forms) of malarial parasites" has been carried out by Mr. Shahid Umar under my supervision. He has fulfilled the requirements of the Aligarh Muslim University, Aligarh regarding the prescribed period of investigational work for the award of M.Phil. degree in Biochemistry.

The work included in this thesis is original unless stated otherwise, and has not been submitted for any other degree.

(M.K. SAHIB)

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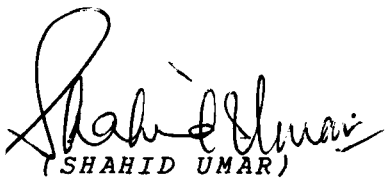


(SHAHID UMAR)

CONTENTS

P R E F A C E

Liver is characterized by its cellular heterogeneity and multiplicity of its biological functions. Most of these functions are expressed in the hepatocyte, although this cell type represents only two thirds of the total liver cell population. Because of this, simple in vitro systems were soon developed to study liver function. Amongst them, isolated perfused liver, tissue slices and subcellular fractions have been widely used for some time. However, short life span of these systems (a few hours) has been one of their most severe shortcomings.

Cultured hepatocytes are the only in vitro model suitable for long term studies. However, it has been observed that hepatocytes lose their differentiated functions after a few days in culture and the cells tend to detach from the culture dishes. The reason for this degenerative course is uncertain, but three factors must principally be considered to be of importance, namely, the absence of other liver cell types, the matrix used for plating the cells and the composition of the medium. Much research has been done to establish the right culture conditions, focussing on the importance of soluble factors (hormones, growth factors and trace elements etc), extracellular matrix components and cell to cell interactions and it is now possible to maintain functional hepatocytes in primary culture for prolonged periods. Experiments presented in this dissertation were aimed at investigating the combined effect of glucocorticoid, insulin and glucagon, as well as DMSO and Se for a period

of 7 days on the survival of hepatocytes, on their plasma membrane integrity and on the maintenance of their differentiated functions. Attempts were also made to use the system for in vitro culture of exo-erythrocytic stage of rodent malarial parasite P. berghei.

From the foregoing, it is obvious that hepatocyte culture has a great potential in understanding the role of other liver cell types in maintenance of differentiated character of the hepatocytes on the one hand and in pathophysiology (once these cell types: endothelial cell, Kupffer cells, and fibroblasts, are injured) on the other. Hepatocytes can also serve as valuable tools for understanding the peptide domains of cytochrome P-450 molelcule that are responsible for substrate specificity of the enzyme. Further, this system has great potential in parasitology in gaining insight into the tissue specificity with regard to differentiation of parasitic forms on the one hand and in understanding mechanism of morbidity/ pathophysiology due to general parasitic infections on the other.

ABBREVIATIONS & SYMBOLS

Ac_2O	: Acetone
BSA	: Bovine serum albumin
CCl_4	: Carbon tetrachloride
CFA	: Complete Fruend's Adjuvant
CPM	: Counts per minute
db_cAMP	: N^6,O^2-dibutyryl adenosine 3',5'-cyclic monophosphate
DMSO	: Dimethyl sulfoxide
EGF	: Epidermal growth factor
EDTA	: Ethylene diamine tetraacetic acid
FBS	: Foetal bovine serum
HEPES	: N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid
mμ	: Millimicrons
mM	: Milli molar
NADH	: Nicotinamide adenine dinucleotide (reduced)
PBS	: Phosphate buffered saline
PAGE	: Polyacrylamide gel electrophoresis
RES	: Reticuloendothelial system
SDS	: Sodium dodecyl sulphate
TP	: Total protein
TCA	: Trichloroacetic acid

INTRODUCTION

One of the primary goals of animal cell culturists over past 50 years has been to achieve long term continuous culture of normal cells exhibiting differentiated properties of their tissue of origin. Despite numerous attempts in many laboratories, progress has been limited in this regard and the occasional successes remain poorly understood. Through most of the history of cell culture, the development of new techniques for survival and growth of cells in vitro has largely been empirical and cell culture in general has suffered from the lack of a unifying approach applicable to novel situations.

Problems raised by both the celullar heterogeneity of the liver and the determination of the role of endogenous and exogenous factors on the different hepatic functions have led investigators to devise in vitro techniques for the study of liver functions in purified cell populations. The techniques used to obtain isolated hepatocytes were originally based on mechanical means, then on the use of chelating agents such as citrate, ethylenediamine tetra-acetic acid (EDTA) or tetraphenylboron to remove the Ca^{2+} and K^{+} which strengthen the intercellular bonds and finally on a treatment using enzyme solutions: trypsin, papain, lysozyme, neuraminidase and pepsin. Under these conditions, the cell yield did not exceed five percent of the total cell population and the functional properties of the majority of the cells were lost (135). A considerable step forward in isolation of intact adult rat hepatocytes was made by the introduction of collagenase and hyaluronidase as dissociating

agents (75). Berry and Friend (16) established the basic protocol involving a two-step perfusion of the liver in situ, first with calcium free buffer, followed by calcium-supplemented buffer containing collagenase. Many authors still use this original protocol which includes Hank's buffer and recirculation of the perfusates. However, a number of investigators have simplified the original technique. The major modifications include omission of oxygenation of the perfusate and the use of HEPES buffer (95). The influence of these various modifications on the cell yield and metabolic activities of freshly isolated rat hepatocytes have been extensively reviewed by Seglen (139). The two-step in situ collagenase perfusion is now a widely used method for obtaining viable dispersed hepatocytes from various species. Moreover, since isolated adult hepatocyte suspensions are a heterogeneous cell population regarding the degree of ploidy and functional activities, several methods have been devised for the separation of different hepatocyte classes.

Primary hepatocyte culture is one of many techniques which simplify the analysis of liver function. The advantage of a culture approach over alternative methods such as isolated liver perfusion is that it permits evaluation of hepatocytes as pure isolates in a controlled environment. Cultures have proven applicable to a variety of experiments, including studies of protein synthesis, intermediary metabolism and enzyme functions (9, 29, 38)and more recently membrane potential and electrical coupling (148, 162). The utility of cultures for intermediate and long term studies has been limited

however, by morphologic and functional alteration of the cells during the first few days after plating and ultimately the deterioration and death of hepatocytes within 1 to 2 weeks (17). Loss of liver specific characteristics reflects the absence in culture of elements that are present and support hepatocellular function in vivo. Research directed at this problem has involved manipulation of the cell culture environment in an effort to define the appropriate conditions under which hepatocytes will maintain a differentiated phenotye for extended periods. Initial studies demonstrated that medium supplements such as nutrients (9, 11) and hormones (21, 42, 52, 53, 54, 79, 89) promote increased protein synthesis and secretion by hepatocyte monolayers. Similarly, introduction of an organic substratum such as Type I collagen or Poly-L-lysine in place of plain tissue culture plastic, enhanced viability and cell attachment. In general, though, simple culture additions have resulted only in improvements which are quantitatively minor or transient. Currently under development are more sophisticated culture methods which introduce factors that may play a role in hepatocellular differentiation in vivo (129)(Table I). Those which rely on medium supplements alone fail to support liver specific functions for more than 1 to 2 weeks (42, 78, 161). On the other hand, cultures that make use of complex extracellular matrix substrata (26, 41,42, 54, 58, 63, 81) have provided dramatic results.

PLASMA PROTEIN PRODUCTION BY CULTURED ADULT HEPATOCYTES

Most plasma proteins with the exception of immunoglobulins are produced by liver. Biochemical and morphological studies have

TABLE I: Putative mediators of hepatocellular differentiation and related cell culture systems.

Factor	Culture	Reference
Soluble mediators (nutrients, hormones, growth factors)	Hormonally defined medium	42, 78, 130
Extracellular matrix	Biomatrix	131
	EHS gel (Matrigel)	136
	Glycosaminoglycans, proteoglycans	148, 162
Cell-cell interactions	Coculture (hepatocytes)	118
	Coculture(nonparenchymal cells)	13, 26, 57, 116

clearly demonstrated that their synthesis is a relatively specialized function of hepatocytes (47). In common with other export proteins, liver plasma proteins are synthesized on bound ribosomes, then conveyed via the endoplasmic reticulum and Golgi apparatus towards the extracellular space (56, 62, 125). Most of them are glycosylated during their transport within the cell. Immunohistochemical analysis suggest that there are no specialized hepatocytes engaged in the formation of one or more plasma proteins in the normal liver. All parenchymal cells can be involved in the production of one or several proteins at the same time (61, 62, 82, 96). However, when the synthesis of some proteins is increased under certain conditions, this increase may not be observed simultaneously in all of the cells. Thus during acute phase response following injection of inflammatory compounds, perilobular hepatocytes are the first to exhibit an increased production of acute phase proteins (28). The production of plasma proteins by the liver is regulated by a variety of endogenous and exogenous factors. In order to understand these regulating factors and particularly their mode of action, a number of investigators have turned to metabolically simpler liver systems and during the last decade have increasingly used freshly isolated or cultured hepatocytes. The work presented in this dissertation reviews studies concerning synthesis of albumin by cultured hepatocytes and underlines the effect of medium composition and extracellular matrix on the rate of albumin synthesis.

USE OF CULTURED HEPATOCYTES FOR XENOBIOTIC METABOLISM AND CYTOTOXICITY STUDIES

(i) Xenobiotic metabolism

Liver plays an important role in handling foreign chemicals, since it is the first organ to receive the chemicals absorbed in the organism. Liver is also the most active mammalian tissue with respect to xenobiotic metabolism (1, 43, 45, 115), and it contains a large variety of enzymes which are able to transform xenobiotics.

Liver is the primary organ involved in the metabolism of xenobiotics. Many compounds are taken up by hepatocytes and converted to pharmacologically inactive, active or toxic metabolites. Xenobiotic metabolism occurs according to various pathways which are classified into two groups. Phase I reactions which include oxidations, reductions and hydrolyses; Phase II conjugation reactions. One major Phase I pathway is represented by the cytochrome P -450 dependent monooxygenases located in the membranes of the endoplasmic reticulum. The monooxygenase system has three main components: a group of hemoproteins collectively called cytochrome P-450, a NADPH requiring flavoprotein (NADPH-cytochrome P-450 reductase) and a lipid moiety which is a constitutive part of the membrane. The multiple forms of cytochrome P-450 account for the wide substrate specificity of the mixed-function oxidase system. Conjugating enzymes (Phase II) are located either in the membranes (UDP-glucuronyltransferase) or in the cytosol (sulfotransferase).

Complex regulatory processes, involving both endogenous and exogenous factors are exerted on hepatic metabolism *in vivo*, and so, a number of investigators have turned to simpler *in vitro* experimental models for studying drug metabolism and toxicity. In particular, isolated and cultured hepatocytes have been increasingly used over the past ten years. Most of the studies have been carried out with rodent cells and the results of these have been extrapolated to humans. However, in view of the qualitative and quantitative interspecies differences which commonly exist in hepatic metabolism of xenobiotics, the validity of such extrapolation is open to question.

Most of the studies have been performed on rat hepatocytes. In conventional culture conditions these cells usually survive for 1-3 weeks (18, 61), but a number of studies have shown that their total cytochrome P-450 rapidly declines. The level is significantly altered within a few hours and is not more than 10 to 40% of the initial value after 24 to 48 hrs of culture (45, 46, 90). The loss of cytochrome P-450 can be temporarily prevented by supplementing the culture medium with a variety of compounds, such as hormones, ligands and heme precursors. A mixture of hormones and nutrients including hydrocortisone, estradiol, testosterone, insulin, thyroxine and fatty acids delays cytochrome P-450 loss for 24 or 48 hrs (34, 36). Paine et al. demonstrated that nicotinamide (123), isonicotinamide (121), metyrapone (90) or methionine in a cysteine-free medium (122) promoted cytochrome P-450 maintenance. Dexamethasone (111), adrenal corticosteroids (112), ascorbic acid (19), 5-aminolaevulinic

acid (34) and glycerol or fructose (64) are also capable of slowing down the decline in the level of cytochrome P-450. Maintenance is not improved by the presence of serum or insulin (46). Other authors cultured rat hepatocytes on organic substrates or in association with other cell types. Michalopoulos et al. (111) measured the level of cytochrome P-450 comparatively in rat hepatocytes cultured on collagen-coated plates, floating collagen gels and on human fibroblasts. They found that cytochrome P-450 was better maintained in cells cultured on human fibroblasts and on collagen gels than in cells cultured on collagen-coated plates. Recently, Begue et al.(13) showed that the cytochrome P-450 level remained relatively stable for atleast 10 days in adult rat hepatocytes co-cultured with another liver cell type. Various inducers (sod. selenite or selenium) may increase the level of cytochrome P-450 and/or several monooxygenase enzyme activities in cultured adult rat hepatocytes. Maximum induction is usually observed following addition of the inducer to 2- or 3-day cultures, the cells being relatively unresponsive during first 24 hr after attachment (91). Some drugs induced a spectrum of cytochrome P-450 forms different from that induced in vivo. Fetal forms are preferentially induced in vitro. This is the case with phenobarbital (46, 90), although as observed in vivo this compound stimulates proliferation of the smooth endoplasmic reticulum in culture. In contrast, other drugs appear to retain the capacity to induce specific forms of cytochrome P-450. In a number of studies cytochrome P-450 was measured by non-specific assay methods which

reflect net changes only of the heme moiety without regard to metabolism of the apoprotein portion. Guzelian's group was the first to demonstrate de novo synthesis of specific forms of cytochrome P-450 after addition of pregnenolone 16-alpha carbonitrile (PCN) and phenobarbital (PB) to nonproliferating rat hepatocytes cultured in a serum free medium. However, the cells contained a lower concentration of total cytochrome P-450 than do freshly isolated counterparts (38). Thus, despite a number of attempts, satisfactory maintenance of cytochrome P-450 levels for several days without changes in the isoenzyme pattern and with preservation of the differential response to inducers of these enzymes has not yet been achieved in rodent hepatocyte cultures. Indeed hepatocytes tend twoards a more fetal-like state and even when normal values of cytochrome P-450 could be maintained for some days, alterations in drug metabolizing enzyme activities were observed suggesting that key regulatory factors remain to be elucidated.

(ii) Cytotoxicity studies

Isolated hepatocytes have become more attractive as a model for the study of drug toxicity. They could also be an excellent approach to problems linked to metabolic interactions between various drugs or drugs and endogenous compounds, or even between drugs and other chemicals found in the environment. Hepatocytes may be used in various ways for toxicological studies. They can also serve for the solution of general cytotoxicity problems such as the

study of a toxic effect at a real molecular level; or to decipher the molecular events linked to the toxicity of a compound:what enzymes, organelles, or other structures are involved and what are the biochemical modifications induced by the chemical? In the study presented here, two parameters have been used to follow general toxicity of the cultured hepatocytes:

(a) Oxygen uptake by viable hepatocytes after they are treated with the compounds, and
(b) release of LDH into the medium.

USE OF CULTURED HEPATOCYTES IN PARASITOLOGY

Many parasites, from protozoans to helminths, have a liver phase in their life cycle. Some simply transit through the liver on their journey to other more permanent sites. However, many may remain there for varying periods of time where they undergo partial or complete maturation. In a few instances, the liver is the site of parasite multiplication, but only rarely do parasites invade parenchymal cell to undergo division. This phenomenon is restricted to a small group of sporozoans belonging to the haemosporididea (Table II).

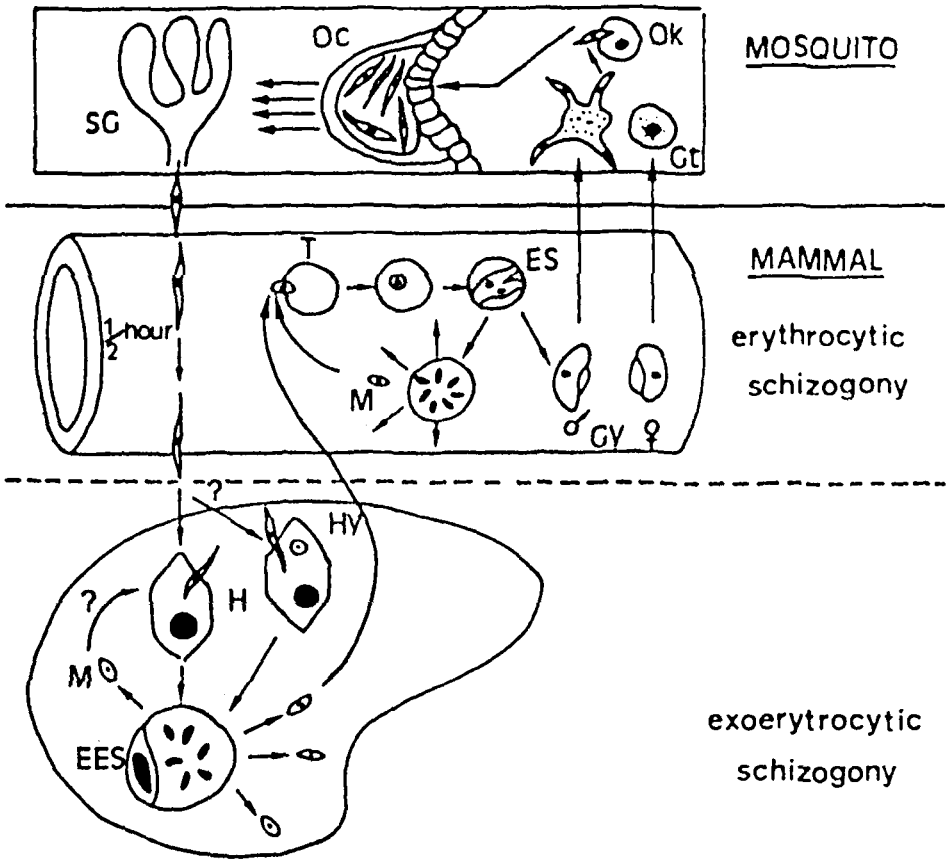
MALARIA

Hepatic stages of mammalian malaria follow the bite of an infected Anopheles mosquito (represented schematically in Fig.1). Injected sporozoites are rapidly cleared from the blood and invade

TABLE II: Parasites for which division in hepatocytes is obligatory or facultative.

Division is obligatory	Division is facultative
Mammalian genera of Plasmodiidae - Genus Plasmodium (Rodent-primate-human) Mammalian genera of haemoproteidae - Hepatocystis (Monkey-bat-squirrel-hyppopotamus-mouse-deer) - Nycteria (Bat) - Biguetiella (Bat)	Leucocytozoidae - Leucocytozoon simondi (duck) multiplication also occurs in macrophages of spleen, liver and bone-marrow

Fig.1: Plasmodium sp cycle (from Gentilini).



EES:exoerythrocytic schizont; S: sporozoite; H: hepatocyte; zm; merozoite; Hy: hypnozoite; T: trophozoite; ES: erythrocytic schizont; Gy: gametocyte; Ok: ookinete; Oc: oocyst; Gt: gamete; SG: salivary glands.

liver parenchymal cells where they undergo a cycle of asexual division (hepatic schizogony). When mature, these exo-erythrocytic schizonts (EES) release merozoites which in turn, may invade red blood cells and initiate repeated cycles of asexual development. Some erythrocytic parasites differentiate into gametocytes, the sexual forms. When a mosquito sucks gametocyte-containing blood, the ingested gametocytes reproduce in the gut where they develop into sporozoites. The cycle is basically same for different plasmodia. However, two human malaria parasites (P.vivax and P.ovale), and some malaria parasite of apes and monkeys cause true relapses for several years. In preference to the continuous cycle theory of Shortt and Garnham (143), there is experimental evidence for the existence in P.cynomolgi (88) and P.vivax (87) of a dormant uninucleated liver form, called hypnozoite.

The hepatic stages, a silent phase of the plasmodia life cycle, were discovered simultaneously by Shortt and Garnham (144) and Hawking et al. (65) in 1948, almost 50 years after erythrocytic stages and sporozoites were discovered by Laveran and Ross, respectively.

Because of its ready availability and clinical relevance, the blood schizogonic cycle has thus far been the most exhaustively studied phase. Furthermore, in vitro cultivation of these stages (66, 154) has led, since 1976, to major improvements in the possibilities of studying these stages. Despite studies on EES in primate hosts and discovery in 1964 of the conditions required for cyclical

transmission of the rodent parasite in the laboratory, intrahepatic schizogony is by far the stage for which our knowledge is the most fragmentary, particularly in human malaria. This is not surprising in view of the difficulty in investigating humans with a parasite located in the liver.

Recourse to animal models has been tempered by a lack of host species susceptible to human malaria parasites. Although it now appears that some species of monkeys, other than those able to sustain blood-infection, may be susceptible to the liver stages (39), this approach has been limited in large part by difficulties in producing sporozoites. Large numbers of sporozoites are necessary for experimental work on this part of the plasmodia life cycle.

HISTORICAL ASPECTS

Culture of the hepatic stages of rodent malaria

In contrast to the avian model in use since 1955 (12), it was only 1978 that rodent pre-erythrocytic forms could be cultivated (51).

As pointed out by Huff in 1963 (76), progress was impeded by the assumptions that EES are rigidly host cell specific and that hepatic parenchymal cells do not grow well in culture. About 10 years later, both these notions were revised, leading to break-through in the cultivation of rodent EES.

The concept of parasite-host cell specificity was greatly undermined in 1974 (12) by successful cultivation of an avian parasite in mammalian cells. This finding illustrated that the presumed host specificity did not necessarily hold true, atleast for *in vitro* systems. It led to the first successful cultivation of EES of a rodent plasmodium, *P. berghei*, in fibroblasts derived from embryonic rat brain and liver, or from embryonic turkey brain (151). Other results subsequently confirmed, the ability of cell lines to support the growth of rodent EES (72), in particular the success in 1981 by Hollingdale et al. in demonstrating complete cycle of *P.berghei* in human embryonic lung cells (70) and later in a hepatoma cell line (71).

The second assumption, the impossibility of cultivating hepatic parenchymal cells, was refuted by improvements in enzymatic isolation and culture of functional hepatocytes (22, 138). These improvements opened the way to cultivating the EES in their natural host cell, the hepatocyte. The first step was the maintenance *in vitro* of liver cells previously infected *in vivo*. By modifying the enzymatic liver dissociation technique of Bonney et al. (22), Foley et al. obtained suspensions of parasitized hepatocytes with viable and infective EES of *P. berghei* (51); these suspensions were utilized to initiate primary cell cultures. Though no morphological entities clearly identifiable as EES were ever observed in the cell cultures, these cultures remained infectious for recipient rodents for upto 44 hrs *in vitro*. This time span corresponds to the incubation period of this parasite in the rat host.

The next step was the initiation *in vitro* of the sporozoite infection achieved in 1981 by Lambiotte et al. (92). Salivary glands of *Anopheles stephensi* containing *P.yoelii* sporozoites were disrupted and added to primary cultures originating from the liver of an adult rat perfused with collagenase solution. Forty-seven hrs after *in vitro* inoculation, numerous well-developed and normal looking schizonts were found in the hepatocyte monolayer. In 1982, the complete *in vitro* cycle was demonstrated by Mazier et al. (105) using hepatocytes of *Thamnomys gazellae*, an African rodent which, *in vivo* is an experimental host for the EES of *P. yoelii*. Viable merozoites were released by the schizonts as demonstrated by the parasitaemia observed in mice infected with culture supernatants. Similar results were obtained by Pirson (126) using rat hepatocytes and *P. berghei* sporozoites isolated by hypaque-discontinuous gradient centrifugation technique and by Meiss et al. who in 1984 described for the first time the ultrastructure of maturing EES of *P. berghei* in primary cultures of Brown Norway rat hepatocytes.

INVOLVEMENT OF KUPFFER CELLS

Shin et al. (142) having observed sporozoites of *P.berghei* within hepatocytes 2 minutes after *in vivo* inoculation, considered this brief lag time as a proof of direct hepatocyte invasion by the sporozoites. Verhave and Meiss (157), however, present both direct and indirect evidence for a prior passage through the Kupffer cells: the existence of an anatomical barrier between the sinusoidal capillary and the hepatocyte (107); the demonstration that blockade

of the RES or destruction of the Kupffer cells result in decreased blood clearance of sporozoites accompanied by a reduction in the number of EES in parenchymal cells (146, 156). Lastly,ultrastructural studies showed that sporozoites, taken up by Kupffer cells, can escape into Disse's space and penetrate into the hepatocytes (104).

Under EES culture conditions, sporozoites do not transit through the Kupffer cells which could contaminate hepatocyte cultures. However, the percentage of sporozoites found intracellularly is low (0.1 to 0.5% with P. falciparum), it is currently impossible to state whether only few of the sporozoites are truly infectious, whether only certain hepatocytes are susceptible to penetration, and whether a transit through a Kupffer cell would increase the infectiousness of the parasite.

MATERIALS AND METHODS

Bovine serum albumin collagenase, insulin, dexamethasone, glucagon, epidermal growth factor, poly-l-lysine, fibronectin, collagen and EDTA were procured from Sigma Chemical Co. USA. L-15, RPMI 1640, MEM, M-199, Penicillin-streptomycin, gentamycin and foetal bovine serum were purchased from Gibco Research Laboratories, USA. Selenium (Sodium salt) and sodium dithionite were purchased from Loba Chemical Co. Bombay. Complete Freund's adjuvant was obtained from Difco, England while (^{14}C)-leucine (specific activity 44 mCi/mmol) was supplied by Bhabha Atomic Research Centre (BARC), Bombay, India.

Dimethyl sulfoxide (DMSO), sodium pyruvate and NADH were purchased from Sisco Research Laboratories, India. All other chemicals and reagents were of A.R. grade.

ANIMALS

Inbred albino rats (Wistar and Sprague Dawly strains) maintained in CDRI Animal House Colony on Hind Lever Pellet diet, Bombay and housed in air conditioned quarters under 12 hours lighting cycle were used throughout the study. Mosquitoes (A.stephensi) were obtained from C.D.R.I. insectory.

ISOLATION OF HEPATOCYTES

Enzymatic liver dissociation provides large numbers of viable hepatocytes which are able to perform many of the functions of the intact liver. Normal rat liver cells were separated from liver by

enzymatic dissociation with collagenase. The method presently used in our laboratory is based on that of Seglen (139). All operations were carried out under sterile conditions. Rats weighing 180-200 gms were anaesthetized with sodium pentobarbitone (Nembutal) and a cannula was introduced into the venous circulation via protal vein. Liver was perfused in situ using a peristaltic pump with two solutions. The first solution, a calcium, magnesium-free HEPES buffer pH 7.65 containing 160.8 mM NaCl, 3.15 mM KCl, 0.7 mM $Na_2HPO_4.12H_2O$ and 0.33 M HEPES, was perfused through the liver at 37°C and at a flow rate of 30 ml/min for 3-5 minutes to remove blood and plasma components and to initiate the cleavage of the desmosomes between liver cells. Liver was then perfused with the same solution to which had been added 0.04 g/100 ml of collagenase and 5 mM $CaCl_2$ at a flow rate of 20 ml/min for 3-5 minutes. When the liver was well-digested, it was removed from the rat by carefully cutting the supporting ligaments and vascular connections, washed with HEPES buffer and placed in L15-Leibovitz enriched medium. The capsule membrane was opened up and the cells were dissociated with the help of blunt edged stainless steel comb. The cells were dissociated further by pipetting several times, filtered through nylon mesh and washed with L15 medium by slow centrifugation at 50 g for 1 min. The cell suspension was then counted by hemocytometer and diluted. The cell yield per liver was observed to be 5-6 ml packed cell volume per liver. Viability of the hepatocytes was checked by 0.2% trypan blue dye exclusion method.

TABLE III: Factors included in the synthetic media.

Factor	Solvent	Final conc.
Insulin	0.01M HCl	10^{-8}M
Glucagon	0.01M NaOH	10^{-10}M
EGF	PBS	10 ng/ml
Dexamethasone	DMSO	10^{-6}M
Thyroxine	0.01M NaOH	10 µg/ml
db cAMP	Dist. water	10^{-4}M
BSA	Dist. water	2%
Selenium (H_2SeO_3)	Dist. water	0.1 µM

Medium was prepared fresh just prior to being added to the plates.

CULTURE CONDITIONS

Hepatocytes were cultured in L15 medium supplemented with penicillin at 100 units/ml and streptomycin at 100 ug/ml. This medium was supplemented with 10% fetal bovine serum and designated serum supplemented medium, or with a defined mixture of trace elements, hormones and growth factors to produce synthetic medium (Table III) (42). A third test medium consisted of a combination of the synthetic medium and dimethyl sulfoxide (Me_2SO).

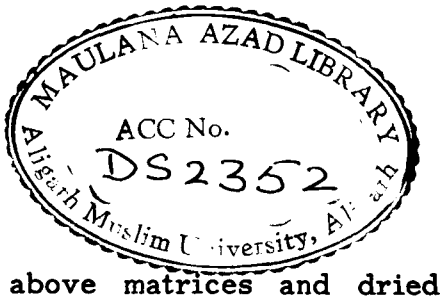
MATRICES USED FOR COATING OF PETRI DISHES TO OPTIMISE PLATING EFFICIENCY

The following matrices were utilized for the coating of the petri plates:

(a) Collagen

(b) Poly-L-lysine

(c) Fibronectin



The plates were coated with the above matrices and dried under UV-light for 2 hours. Later on, the plates were placed in a vacuum dessicator and dried in vacuo over fused calcium chloride at 37°C for atleast 6 hours. The cells not sticking were removed, dissociated and counted by hemocytometer. The plating efficiency using each of the three matrices is summarised in the result section. After six hours incubation at 37°C, hepatocyte monolayers were fed a synthetic medium and the incubation was continued overnight. At 20-24 hrs after plating, cultures were fed synthetic medium

supplemented with dimethyl sulfoxide (DMSO) and fed every 24 or 48 hr for the remainder of the experiment.

DEVELOPMENT OF IN VITRO MODEL OF HEPATOTOXICITY

Two parameters were taken to develop an in vitro model of hepatotoxicity from the hepatocytes cultured in synthetic medium and subjected to treatments of different hepatotoxic compounds. These parameters are:

(a) determination of lactate dehydrogenase leakage from the cells into the medium and

(b) measurement of O_2 uptake by hepatocytes

The above parameters were also used to evaluate the efficacy of various natural plant products/other compounds on prevention or inhibition of toxic manifestation due to the toxic insults.

(a) Assay of lactate dehydrogenase

Lactate dehydrogenase activity was determined (according to the procedure of Kornberg, 1955) (86) in the Beckman spectrophotometer at 340 mu. One tenth to 0.5 ml of appropriately diluted enzyme was added to a cuvette with a light path of 10 mm containing 0.1 ml of 0.0025 M NADH, 0.3 ml of 0.01 M sodium pyruvate and M/15 phosphate buffer to a final volume of 3 ml. The final concentration of pyruvate used was necessary to obtain optimal activity for a given amount of enzyme. One unit of enzyme activity was defined

as an amount which causes a decrease in absorbance by 0.001 in one minute at 25°C-28°C. Initial velocities were determined during the zero order portion of the reaction.

Total LDH activity was measured by following the penetration of NADH into the cells using LDH latency test : an aliquote of a well-mixed hepatocyte suspension was diluted 20-fold in phosphate buffer, pH 7.3, containing 2% albumin, NADH (0.1 mM final conc) and pyruvate (0.76 mM final conc) were then added. The rate of NADH oxidation was recorded at 340 mu, and 100% activity was obtained after lysis of the cells by the addition of Triton X-100 (0.5% final concentration).

(b) Oxygen uptake measurements

Oxygen consumed by the cells was measured with the help of Gilson's oxygraph with sensitivity fixed at 160 units and a chart speed of 0.1 mm/second. Oxygen uptake was expressed as μmoles of oxygen consumed/min/mg protein (equivalent to 10^6 cells).

PREPARATION OF ANTI-NORMAL ADULT RAT SERUM ALBUMIN

Four subcutaneous injections of 2, 4, 4, 6 mg of the purified rat serum albumin in 1.0 ml of normal saline mixed with equal volume of complete Freund's adjuvant was injected to the rabbit subcutaneously at a one week interval. Then, three booster doses of 6 mg each were administered to the animals intramuscularly. Finally they were bled on the 10th day after the last injection

when the antibody titre had peaked as was revealed by the double diffusion analysis. Albumin was purified by DEAE-cellulose chromatography and characterized by SDS-gel electrophoresis. Albumin-content was determined by rocket immunoelectrophoresis (Fig. 2).

SYNTHESIS AND SECRETION OF ALBUMIN BY HEPATOCYTES IN CULTURE

^{14}C-leucine incorporation (3 μCi/ml) into newly synthesized and secreted albumin by hepatocytes in culture was followed by immunoprecipitation of labelled albumin in culture medium (synthesis and secretion). Aliquots of 100,000xg cell free supernatant and culture medium were mixed with 100 ul of normal rat serum (1:10 dilution) to provide optimal carrier for the immunoprecipitation of the labelled albumin. Albumin was then precipitated by addition of 0.5 ml of rabbit anti-rat serum albumin, and the mixture was incubated at 37°C for 1 hr and at 4°C overnight for proper precipitation. The immunoprecipitate was collected by centrifugation at 4,000 rpm for 15 min in cold centrifuge. The immunoprecipitates thus obtained were washed several times with PBS containing 0.1 ml leucine (PBS-leucine) to remove nonspecific adsorption of ^{14}C-leucine. Finally the immunoprecipitates were dissolved in 0.4 ml of 0.1 N NaOH. Radioactivity was determined in 0.2 ml of aliquots in a Tricarb Liquid Scintillation Counter (Packard Model 3330).

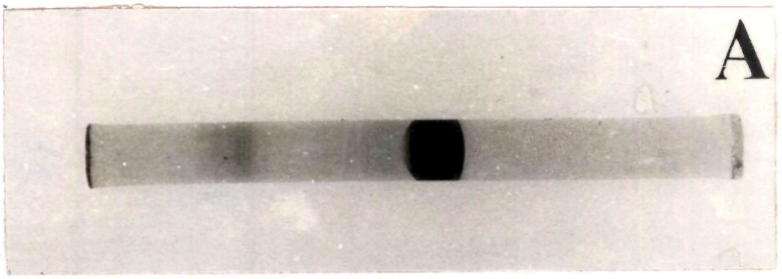
DETERMINATION OF TOTAL PROTEIN SYNTHESIS AND SECRETION

Total intracellular protein synthesis (in 100,000 x g cell free supernatant) and secretion (in culture medium) was determined by following

Fig. 2 A) SDS-PAGE of rat serum albumin, Electrophoresis was performed on 7% gels for 2 hr at a constant current of 1.5 mA/tube.

B) Pattern of rocket assay of albumin.

C) Ouchterlony's double diffusion analysis using 1% agarose. The plate was allowed to diffuse for 24 hrs, washed, dried and stained with amido black.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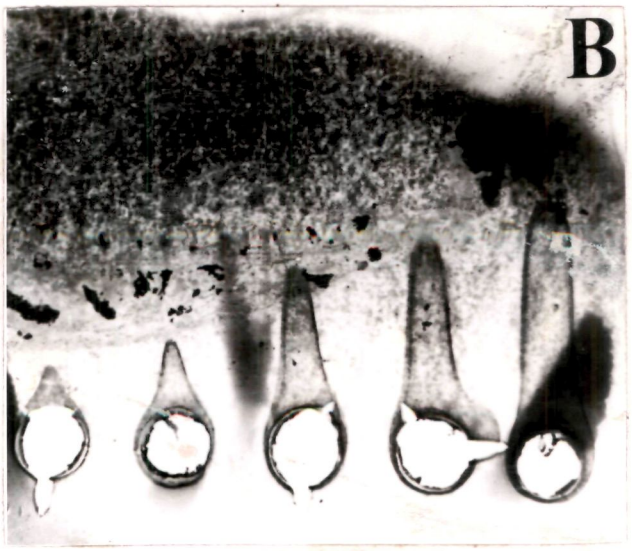
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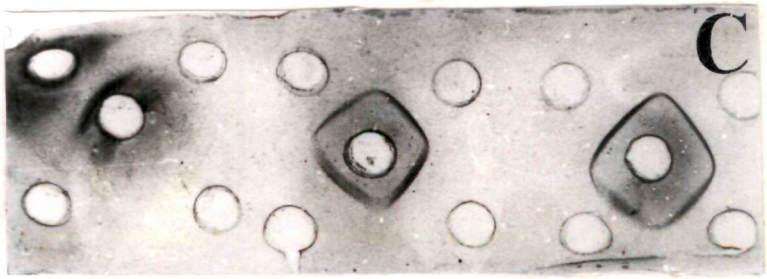

C

the incorporation of (^{14}C)-leucine (3 μCi/ml) into 100% TCA precipitable fractions. Aliquots of cell free supernatant and culture medium (0.5 ml each) were mixed with 100 ul of normal rat serum (diluted 10 times) to provide carrier-proteins and the proteins were precipitated by the addition of 0.6 ml of 10% TCA. The mixture was shaken thoroughly and then allowed to stand at 4°C for 3 hours. The precipitate was collected by centrifugation at 4,000 rpm for 15 minutes, then washed several times with 5% cold TCA containing 0.10% leucine and finally dissolved in 0.4 ml of 0.1N NaOH. Radioactivity was determined in 0.2 ml aliquots in a liquid scintillation counter.

CYTOCHROME P-450 MEASUREMENT

Cytochrome P-450 levels were assyed in the microsomal fractions of the cultured hepatocytes prepared by differential centrifugation.

Microsome preparation

Microsomes were prepared by differential centrifugation from a group of 5 culture dishes. After two washes with HEPES buffer pH 7.4, hepatocyte cultures were scraped with a rubber policeman and pelleted. The cell pellets were homogenized with a Potter Elvejhem homogeniser in 50 mM sodium phosphate buffer pH 7.4 containing 1 mM EDTA. Cell homogenate was centrifuged at 800 g for 10 minutes, at 13,500 g for 20 minutes and at 100,000g for 60 minutes. The pellet obtained after ultracentrifugation was suspended in 50 mM sodium phosphate buffer (pH 7.4).

Assay

Cytochrome P-450 level was estimated according to the method of Omura and Sato (1964) (120). The base line was determined by placing 2.5 ml microsomal suspension in each of two matched cuvettes (1 cm light path) and recording the spectrum from 500 to 400 nm using Shimadzu double beam spectrophotometer (Model UV-90). About 5 mg sodium dithionite was added to the sample cuvette and the difference spectrum between the reduced and oxidized cytochromes was recorded at the same wavelength. Now 5 mg of solid sodium dithionite was added to the reference cuvette and the contents mixed thoroughly, carbon monoxide was bubbled gently into the sample curvette and the difference spectrum was once again recorded from 500 to 400 nm. The amount of cytochrome P-450 was calculated from the difference in the absorbance (450-480 nm) and the molar extinction coefficient of 91 $cm^{-1}mM^{-1}$. Microsomal protein content was measured by the method of Lowry et al. (100).

PARASITOLOGICAL STUDIES

DEVELOPMENT OF THE EE STAGES OF RODENT MALARIA PARASITE, P.BERGHEI IN VITRO

Parasite strain — Plasmodium berghei NK-65

Vector: — Anopheles stephensi

Gametocyte donors: — Mosquitoes (Anopheles stephensi) were allowed to feed on the Mastomys 3-days after the mastomys were infected with P.berghei sporozoites.

Rate of infection of mosquitoes: The number of oocysts in the midgut first checked at day 7 after the infective meal. It has been observed that very large number of oocysts produce a low rate of infective sporozoites. The optimum number developing into schizonts is obtained from between 20 and 100 oocysts. Cages of mosquitoes used to infect the cultures were selected accordingly.

INFECTION OF CULTURES WITH SPOROZOITES

Infected salivary glands were dissected asceptically, pooled and disrupted by trituration in a glass tissue grinder. The sporozoites were counted and an appropriate inoculum (40,000 sporozoites per 10^6 hepatocytes) was added to each culture plate containing minimum of medium. Three hours later, further culture medium was added. After 48 hrs the cultures were rinsed three times in phosphate buffered saline (PBS) pH 7.5, fixed for 10 min at room temperature in methanol and again washed three times. They were then stained with Giemsa (1:10 solution in phosphate buffer, pH 7.4) and examined under microscope.

RESULTS

1. CULTURE OF HEPATOCYTES

(a) Viability of cells after obtaining suspension

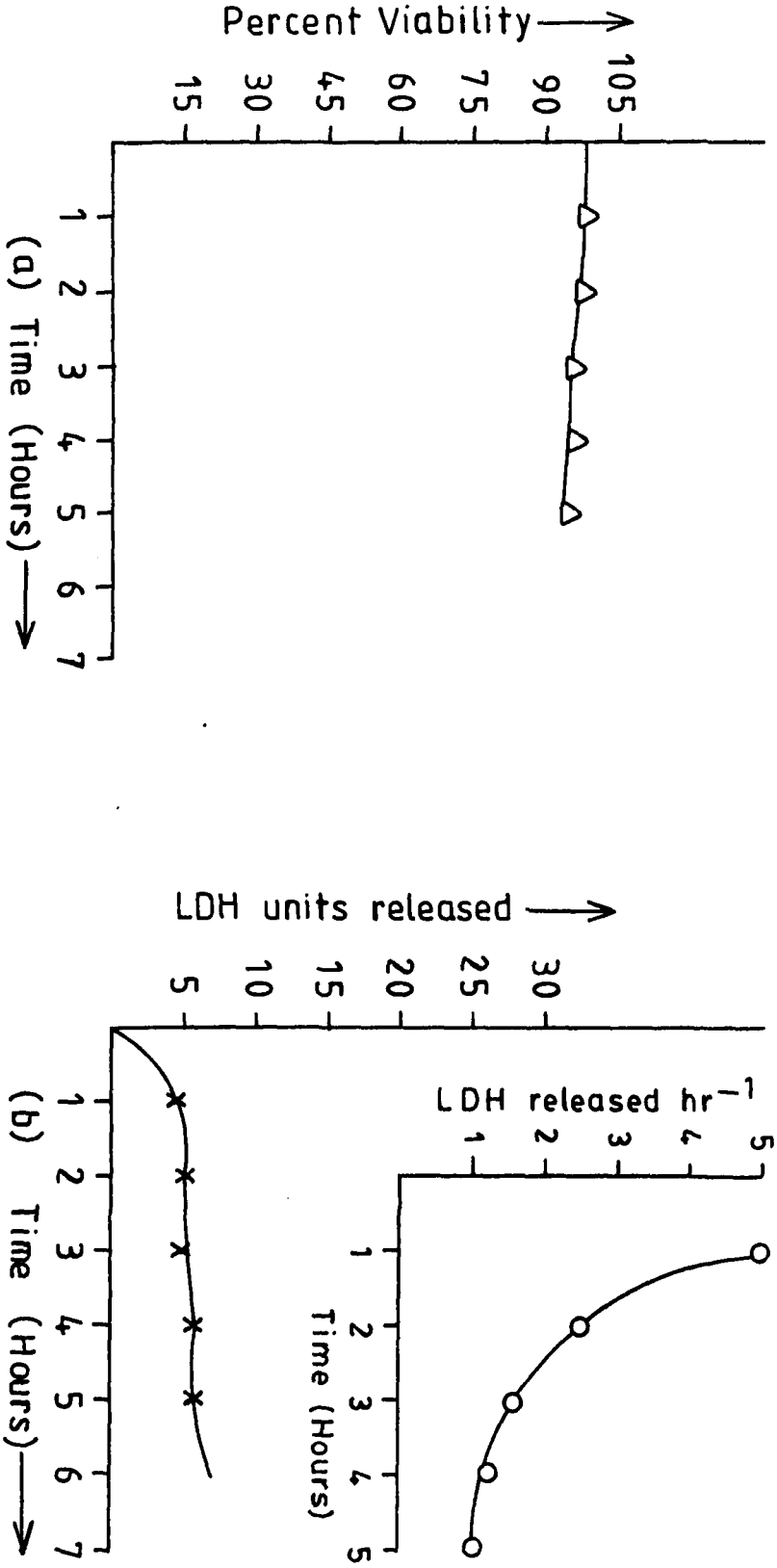
Isolation of rat hepatocytes by in situ perfusion of liver with collagenase yielded viable cells in high quantities. The viability and integrity of the cultured cells were assessed by trypan blue dye exclusion, measurement of total lactate dehydrogenase activity as well as leakage of the same from the cells into the medium. Ninety percent or more of the cells excluded trypan blue and LDH leakage was minimal. Upon incubation of the hepatocytes in a synthetic medium for upto 6 hour, trypan blue exclusion frequency showed a small decrease while intracellular LDH levels and LDH leakage into the medium showed plateauing off. It appears, therefore, that after initial shock due to collagenase digestion associated tissue dissociation, the cells appear to have reversed steadily as a function of time as can be observed from the slope of the LDH units released into the medium as a function of time of incubation (Fig. 3).

(b) Plating efficiency

The plating efficiencies obtained with the three matrices, collagen, poly-lysine and fibronectin are given below.

(i) **Collagen:** Collagen was extracted from rat tail tendons in 0.5M acetic acid at $4^{\circ}C$ for 1-2 days (150). The solution was then passed through muslin to remove insoluble material. The solution was then dialyzed for 24 hrs and centrifuged. The clear solution obtained was sterilized under U.V. light and used for coating plates and

Fig.3: Viability parameters of hepatocytes isolated from adult rat during incubation in a synthetic medium.


Percent Viability
15
30
45
60
75
90
105
(a) Time (Hours)
1
2
3
4
5
6
7
LDH units released
5
10
15
20
25
30
LDH released hr⁻¹
1
2
3
4
5
(b) Time (Hours)
Time (Hours)

preparing collagen gels on nytex. Collagen coating improved plating efficiency of hepatocytes from 30% to 70%.

(ii) Poly-l-lysine: When coated on the plastic petri dishes at a concentration of 2.5 to 5 $\mu g/cm^2$ yielded 80% plating efficiency.

(iii) Fibronectin: Fibronectin when used at a concentration of 2-5 $\mu g/cm^2$ yielded a plating efficiency of 50%.

(c) Morphology of hepatocytes in presence of additives which improve hepatocyte culture

When cultured in presence of synthetic medium and factors that improve cell growth or differentiation, hepatocytes were observed to undergo morphological changes upon seeding and subsequent culture on various substrata. The synthetic medium as reported for hepatocyte culture by Enat et al. (42) with slight modification and found to be effective is presented in Table III. Hepatocytes seeded in the synthetic medium supplemented with fetal bovine serum (FBS) reaggregate within a few hours, spread and form typical monolayers of continuous granular cells after 2-day in culture (Fig. 4A). Spreading was delayed in a serum-free medium. Addition of Se (0.1 μM) slightly reversed this delay. Marked differences were observed in serum-free medium deprived of cyst(e)ine: the cells attached but did not spread normally. In addition in the absence of cyst(e)ine, the cells tended to detach readily from the substratum. Cells also began to detach by day 7 when they were seeded onto tissue

culture plastic dishes used without coating with any matrix. Cells plated on collagenous or poly-l-lysine substrata were attached stably throughout the two weeks of the experiments. Spreading was also impeded when DMSO (5.6×10^{-4}M) was added to the synthetic medium. However, addition of DMSO improved plating efficiency and helped to maintain parenchymal cells in viable and differentiated state for more than two weeks (Fig.4B).

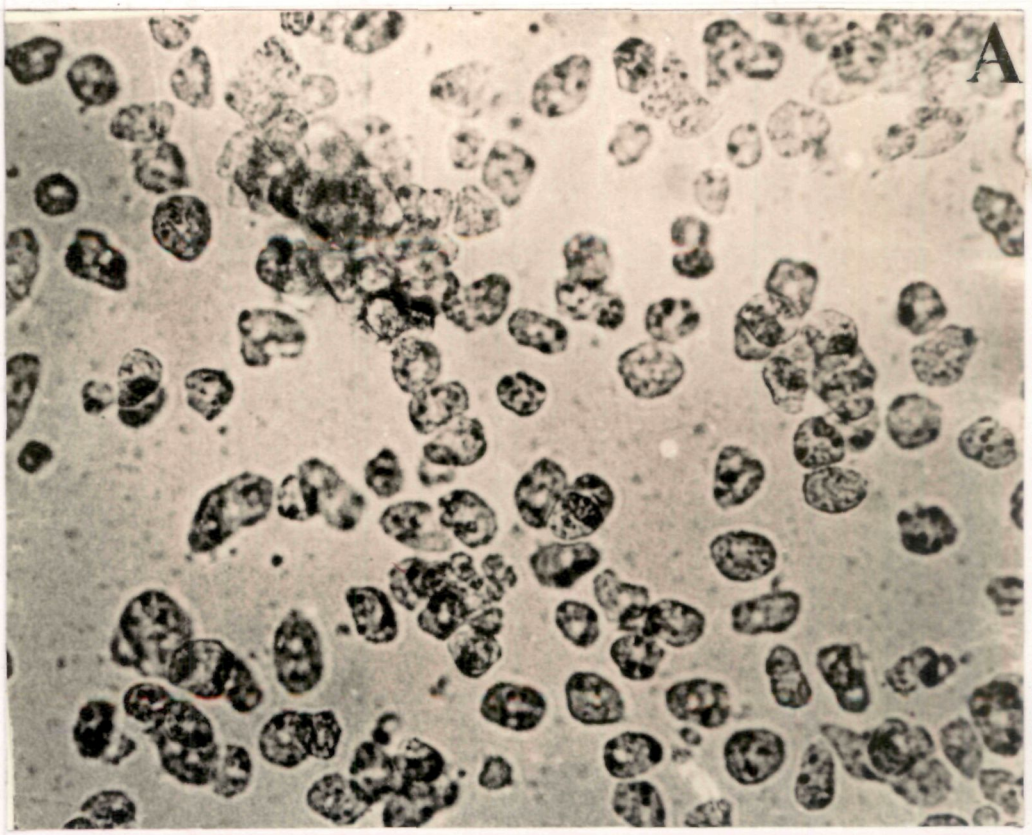
2. DEVELOPMENT OF IN VITRO MODEL OF HEPATOTOXICITY

(a) Hepatotoxic agents

Hepatocyte cultures in presence of synthetic medium were used as an experimental model for toxicity assessment. Cytotoxicity was evaluated by following integrity of the plasma membrane by measurement of cytosolic enzyme leakage into the medium, oxygen uptake and determination of cell viability by the trypan blue exclusion. Two cytotoxic agents, acetone and carbon tetrachloride, were tested for their ability to induce hepatotoxicity in the cultured hepatocytes. The extent of damage caused due to acetone and carbon tetrachloride addition was measured by following the release of lactate dehydrogenase into the medium and oxygen consumption by the hepatocytes. It was observed that while both acetone and CCl_4 caused increased leakage of LDH into the medium and reduced O_2 uptake, the two had a synergistic effect when they were used in combination. Fingure (5) shows the standardization of the acetone concentration required to produce 50% inhibition of oxygen uptake.

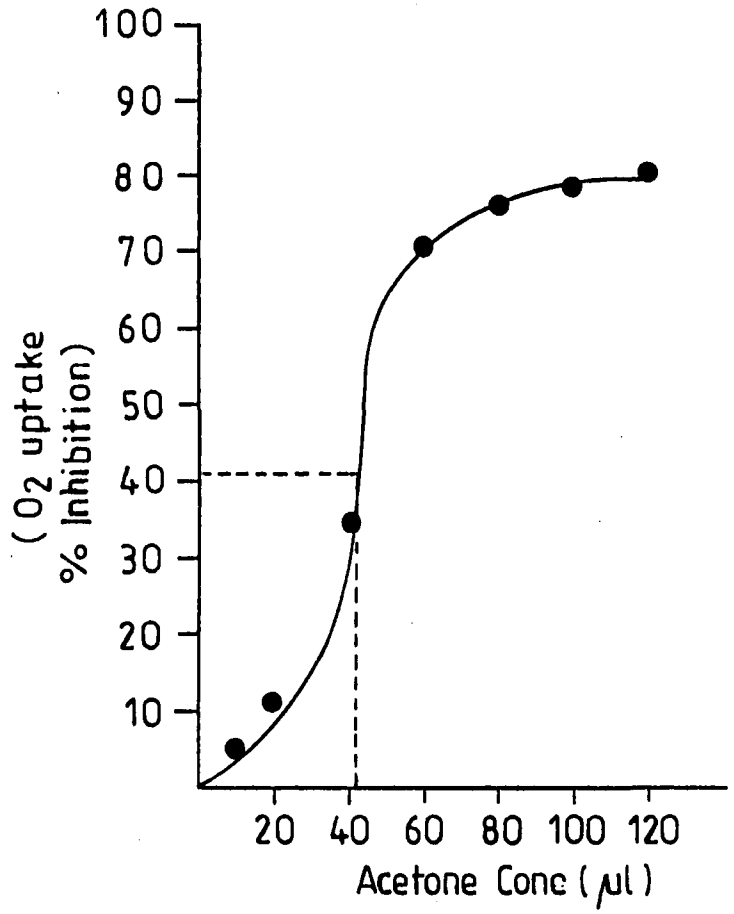
Fig. 4A: Primary cultures of normal adult rat hepatocytes on poly-l-lysine coated plates in serum supplemented synthetic medium.

B) Scanning E-M's of adult rat hepatocytes cultured in DMSO supplemented synthetic medium on poly-l-lysine coated plates.


A


10 µm 25.1 kV 2.20E3 0131/03 SE

Fig. 5 Concentration of acetone required to produce 50% inhibition of oxygen uptake (IC_{50} value).


100
90
80
70
60
50
40
30
20
10
(O_2 uptake
% Inhibition)
20
40
60
80
100
120
Acetone Conc (µl)

(b) Hepatoprotective agents

To overcome the toxic effect of acetone and CCl_4 on the metabolism of cultured hepatocytes, nutrient medium was supplemented with dimethyl sulfoxide and selenium. These two proved to be the best hepatoprotective agents.

The following experiments were conducted to explain the mechanism of acetone/CCl_4 toxicity and its protection by DMSO.

LACTIC ACID DEHYDROGENASE LEVELS AND OXYGEN UPTAKE MEASUREMENT IN PRESENCE OF ACETONE/CCl_4 AND DMSO

Release of lactate dehydrogenase into the culture medium and the oxygen uptake by the cells was determined in the control and after the hepatocytes were incubated with the following compounds.

(a)	Acetone	($5x10^{-4}$M final conc)
(b)	CCl_4	($4.1x10^{-4}$M final conc)
(c)	Acetone:CCl_4	[$(2.5:2.5)x10^{-4}$M final conc)
(d)	DMSO	($5.6x10^{-4}$M final conc)
(e)	Acetone:CCl_4+DMSO	[$2.5:2.5)x10^{-4}$ + $5.6x10^{-4}$M final conc)

After O/N culture at 37°C in the synthetic medium, cells were incubated with the above compounds at the specified concentration. The incubation was carried out for 0, 2, 4 and 6 hrs. After stipulated time, the cells were centrifuged, supernatant was saved for LDH assay while the cells were utilized for O_2 uptake measurements (Table IV & V).

TABLE IV: Effect of DMSO on LDH levels and oxygen uptake measurements in presence of acetone/CCl_4.

S.No.	Addition	LDH units leaked from 10 cells in cultured medium	O_2 uptake (μmol/min/mg protein)
1.	Control	86±5	0.24±0.03
2.	Acetone	195±6	0.09±0.05
3.	CCl_4	210±6	0.06±0.06
4.	Acetone+CCl_4 (1:1)	310±8	0.02±0.01
5.	DMSO	102±5	0.21±0.03
6.	Acetone+CCl_4+DMSO	124±6	0.18±0.04

Results are mean ± SD derived from 3 independent experiments.

TABLE V: LDH levels and O_2 uptake in presence of acetone/CCl_4 and DMSO at different time intervals.

Addition	Time of incubation							
	0 hr		2 hr		4 hr		6 hr	
	LDH leakage (units released /10^6 cells)	O_2uptake (µmol/ min/mg protein	LDH leakage (units released/ 10^6 cells)	O_2uptake (µmol/ min/mg protein	LDH leakage (units released/ 10^6 cells)	O_2uptake (µmol/ min/mg protein	LDH leakage (units released/ 10^6cells)	O_2 uptake (µmol/ min/mg protein
1. Control	95±4	0.21±0.03	98±3	0.20±0.03	101±2	0.20±0.03	106±4	0.29±0.04
2. Acetone	148±5	0.11±0.06	195±6	0.09±0.04	210±6	0.08±0.04	251±6	0.06±0.05
3. CCl_4	166±6	0.09±0.05	216±7	0.06±0.06	265±5	0.04±0.04	295±6	0.02±0.02
4. Acetone+CCl_4	238±5	0.06±0.06	319±8	0.04±0.03	433±8	0.02±0.02	443±7	0.01±0.01
5. DMSO	102±3	0.19±0.03	98±5	0.18±0.03	105±6	0.16±0.02	105±5	0.16±0.03
6. Acetone+CCl_4 +DMSO	114±4	0.18±0.03	119±5	0.14±0.04	123±3	0.16±0.03	124±5	0.14±0.03

Results are mean ± SD derived from 3 independent experiments.

3. NATURAL PLANT PRODUCTS AS AGENTS THAT PROTECT HEPATOCYTE PLASMA MEMBRANE

Hepatoprotective effect of some natural plant products was observed by following the inhibition of leakage of lactate dehydrogenase from the O/N cultured hepatocytes into the medium, and the oxygen consumed by the cells recovered from the culture dishes. The plant products were dissolved in H_2O/DMSO to a final concentration of 15 ug/ml and filtered through millipore filters. The cells were incubated with the compounds for one hour at 37°C. DMSO controls were prepared separately. After one hour post incubation, 40 ul ($5x10^{-4}$M) of acetone was added to each plate and the cells were further incubated for 2 hrs. The LDH activity was then followed in a 20,000xg supernatant while oxygen uptake measurement was carried out in the cells in presence of glucose. It was observed that out of 44 compounds tested for hepatoprotective activity only 16 showed some ability to protect hepatocyte membrane. Amongst them, eight compounds were strongly hepatoprotective (Table VI).

4. OXYGEN UPTAKE MEASUREMENTS

Integrity of the hepatocyte membrane was determined by measuring the micromoles of oxygen consumed by the cells when cultured in a synthetic medium containing DMSO and Se.Oxygen uptake measurements were carried out from:

a) i) fresh hepatocytes,

ii) hepatocytes maintained in culture for seven days and

TABLE VI: Effect of hepatoprotective plant extracts on acetone induced impairment of hepatocyte plasma membrane: increased LDH leakage and inhibition of O_2 uptake.

S.No.	Addition	LDH (Units released/10^6 cells/2h)	O_2 uptake (µmol/min/mg protein)
1.	Control	129±6	0.21±0.03
2.	Acetone (Ac_2O)	210±8	0.08±0.02
3.	Ac_2O+TR/10	140±4	0.18±0.03
4.	Ac_2O+TR/17	70±5	0.24±0.06
5.	Ac_2O+TR/16	100±5	0.22±0.04
6.	Ac_2O+TR/44	180±6	0.09±0.02
7.	Ac_2O+TR/70	140±4	0.16±0.03
8.	Ac_2O+TR/37	120±4	0.20±0.06
9.	Ac_2O+TR/43	120±3	0.20±0.05
10.	Ac_2O+TR/46	121±4	0.21±0.05
11.	Ac_2O+TR/74	200±8	0.06±0.02
12.	Ac_2O+TR/77	78±6	0.22±0.06
13.	Ac_2O+TR/78	78±5	0.024±0.04
14.	Ac_2O+TR/82	150±6	0.09±0.02
15.	Ac_2O+TR/83	170±8	0.06±0.01
16.	Ac_2O+TR/88	172±7	0.06±0.02
17.	Ac_2O+TR/94	119±4	0.18±0.04
18.	Ac_2O+TR/95	122±4	0.16±0.04

Results are mean ± SD derived from 3 independent experiments.

b) hepatocytes maintained for seven days in DMSO and Se in addition to synthetic medium.

The cells were scraped with a rubber policeman and centrifuged. The supernatant was discarded. To the pellet, 2 ml of nutrient medium was added and the suspension obtained was utilized for oxygen uptake measurement. The results are expressed as umoles of O_2 consumed/min/mg protein. After 7 days culture in synthetic medium substantial amount of cells were viable and consumed measurable oxygen while the values were much higher in presence of DMSO and Se(Table VII).

5. SYNTHESIS AND SECRETION OF ALBUMIN BY HEPATOCYTES IN CULTURE

Synthesis and secretion of albumin by the hepatocyte in culture was studied by incubating the cells with 2 ml of leucine-free medium supplemented with hormones, trace element and antibiotics in addition to DMSO at 37°C in a CO_2-incubator. Experiments were initiated by adding ^{14}C-leucine to the culture medium (3 μCi/ml). Incubation was terminated after three hours by removing the plates from the incubator and chilling them on ice-bath. The cells were centrifuged at 4000 rpm for 15 minutes and subsequently the incubation medium was removed. The cells were then washed several times with phosphate buffered saline containing 0.1% DL-leucine (PBS-leucine) and finally homogenized in motor driven Potter Elvehjem homogenizer using a teflon coated pestle.

TABLE VII: Oxygen uptake measurement in presence of a) synthetic medium and b) synthetic medium + DMSO & Se.

S.No.	Preparation	O_2 uptake (μmol/min/mg protein)	
		(a)	(b)
1.	Freshly isolated cells	0.22±0.04	0.21±0.03
2.	1-day culture	0.20±0.04	0.20±0.03
3.	2-day culture	0.16±0.03	0.14±0.02
4.	3-day culture	0.10±0.04	0.18±0.3
5.	5-day culture	0.06±0.02	0.15±0.3
6.	7-day culture	0.09±0.03	0.12±0.4

Results are mean ± SD derived from 3 independent experiments.

Total protein and albumin synthesis was followed by monitoring rate of incorporation of ^{14}C-leucine into TCA precipitable and immunoprecipitable (using monospecific rabbit anti-rat albumin) fractions of 100,000xg supernatant of rat hepatocyte homogenate in phosphate buffered saline pre-exposed to ^{14}C-leucine pulse for 3 hr. Similarly the rate of secretion of total protein and albumin was followed by monitoring the amount of radioactivity in TCA precipitable and immunoprecipitable fractions respectively. Addition of 5.6×10^{-4}M DMSO to the synthetic medium had positive effect on rate of albumin synthesis by hepatocytes. The results are summarized in Fig. 6 and 7.

6. CYTOCHROME P-450 MEASUREMENT

Cytochrome P-450 was measured immediately after isolation of the hepatocytes prior to plating and after 1, 3, 5 and 7 days in culture (Fig.9). Whole liver microsomes prepared by ultracentrifugation were used as controls. Hepatocytes grown for 3 days or longer in synthetic medium lacking cysteine had substantially higher levels of cytochrome P-450 than hepatocytes grown in defined medium containing cysteine. In fact there appeared to be an induction of cytochrome P-450 when the hepatocytes were incubated in the cysteine-free medium for three days. Cytochrome P-450 levels were nearly twice those of the freshly isolated cells or the cells grown for 3-days in the medium supplemented with cysteine.

7. DEVELOPMENT OF EXO-ERYTHROCYTIC SCHIZONTS

When the cultures were stained with Giemsa, they revealed

Fig. 6: ^{14}C-Leucine incorporation into albumin (ALB) and total protein (TP) by hepatocytes cultured for 24 hr in synthetic medium.
Fig. a (synthesis) and b (secretion) represents radioactivity found in the proteins.
Results are mean ± SD derived from 3 independent experiments.

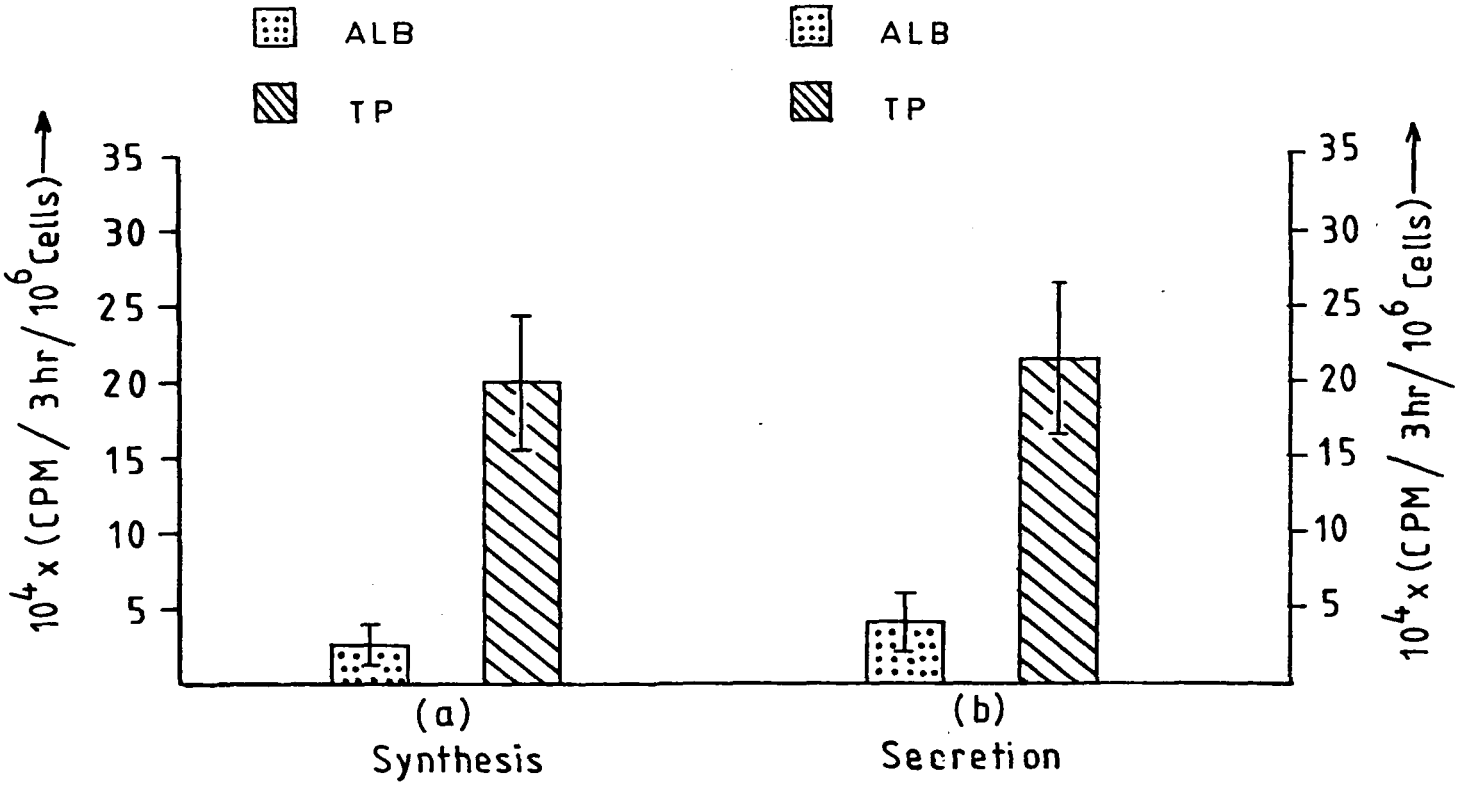

ALB
TP
ALB
TP
10^4 x (CPM / 3 hr / 10^6 Cells)
35
30
25
20
15
10
5
(a)
Synthesis
(b)
Secretion

Fig. 7: ^{14}C-Leucine incorporation into albumin (ALB) and total protein (TP) by hepatocytes cultured for 24 hr in synthetic medium + DMSO.
Fig. a (synthesis) and b (secretion) represents - radioactivity found in the proteins.
Results are mean ± SD derived from 3 independent experiments.

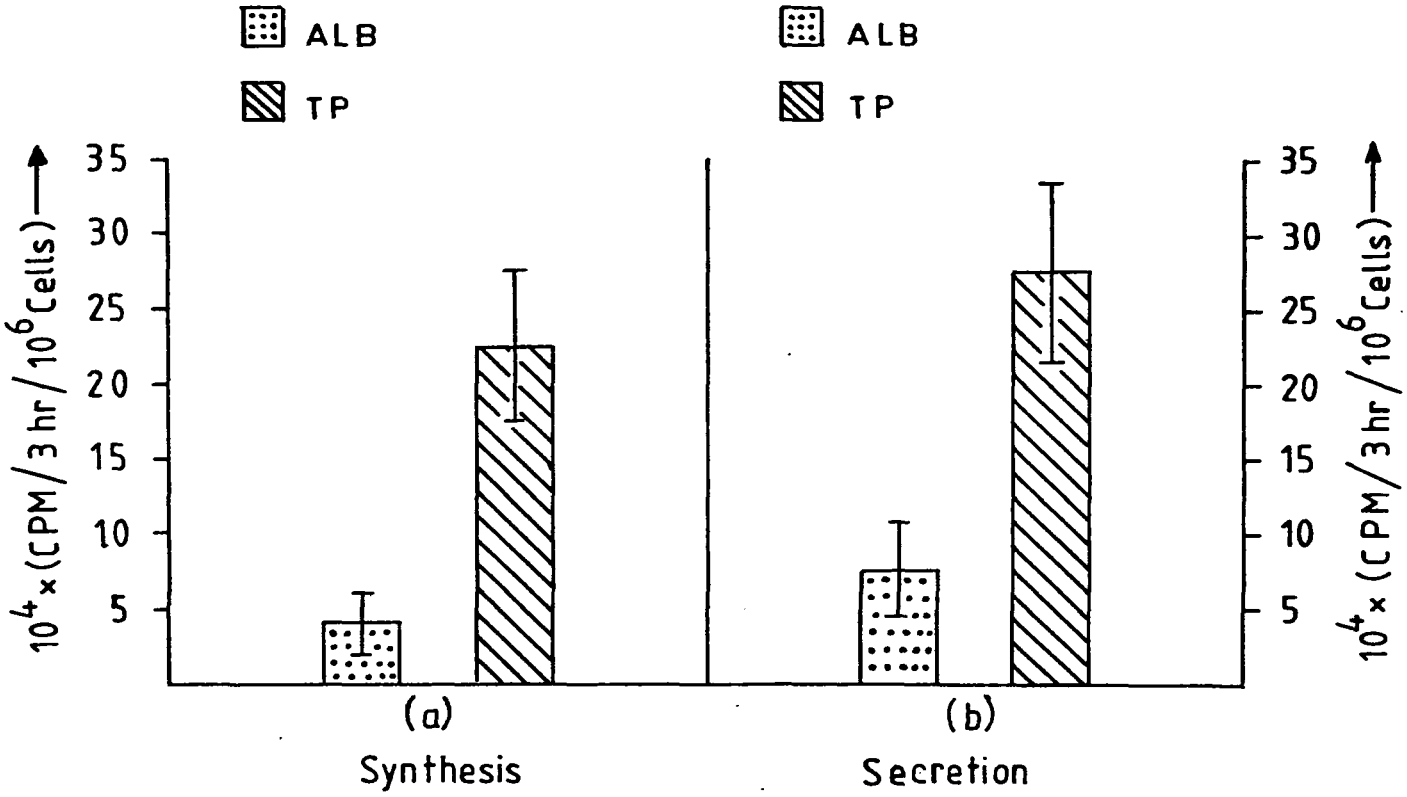
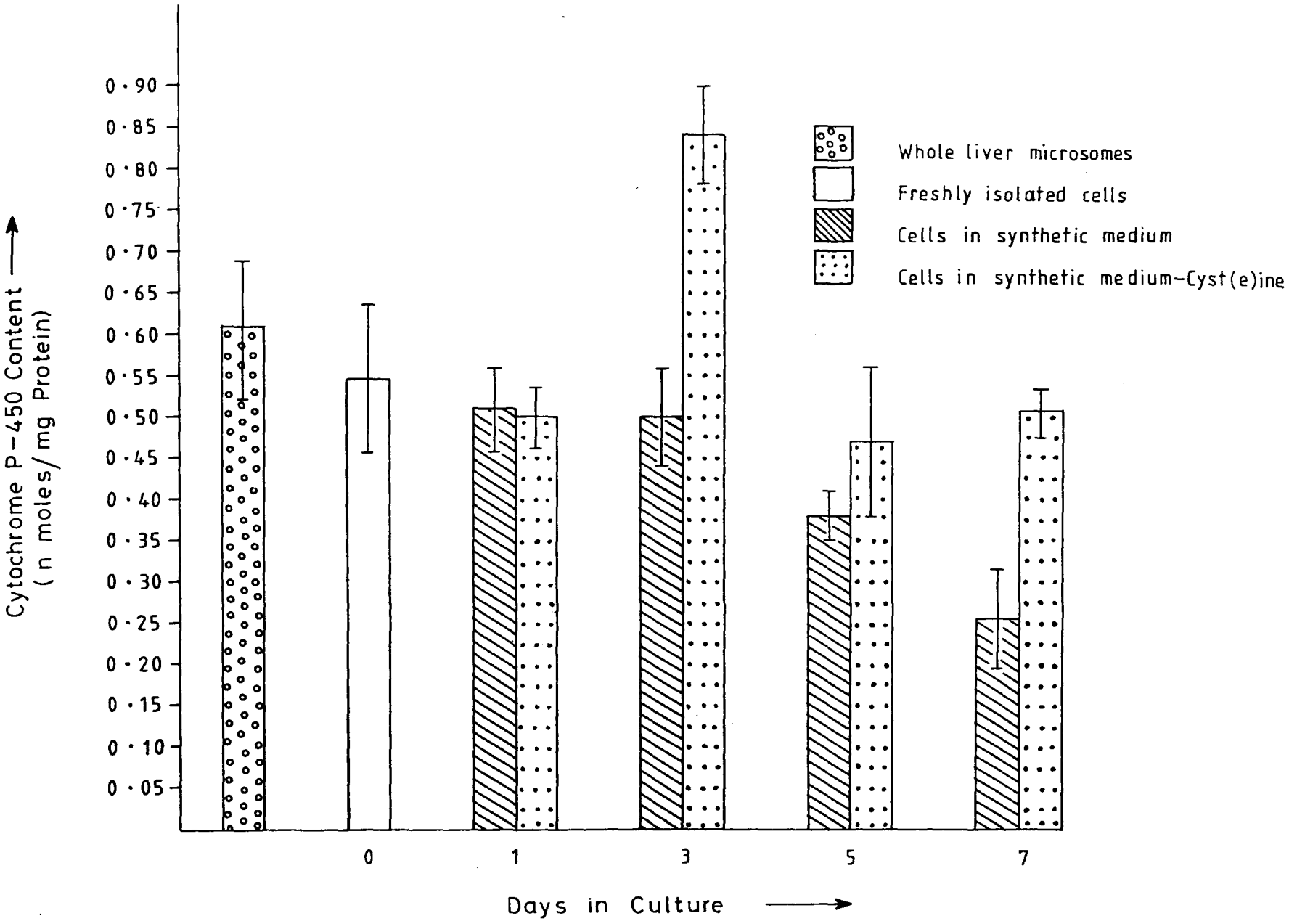

ALB
TP
ALB
TP
35
30
25
20
15
10
5
$10^4 \times$ (CPM/3 hr/10^6 Cells)
35
30
25
20
15
10
5
$10^4 \times$ (CPM/3 hr/10^6 Cells)
(a)
Synthesis
(b)
Secretion

Fig.8: Levels of microsomal cytochrome P-450 in freshly isolated hepatocytes - from adult rats and in primary cultures of adult rat hepatocytes.
Results are mean ± SD derived from 3 independent experiments.

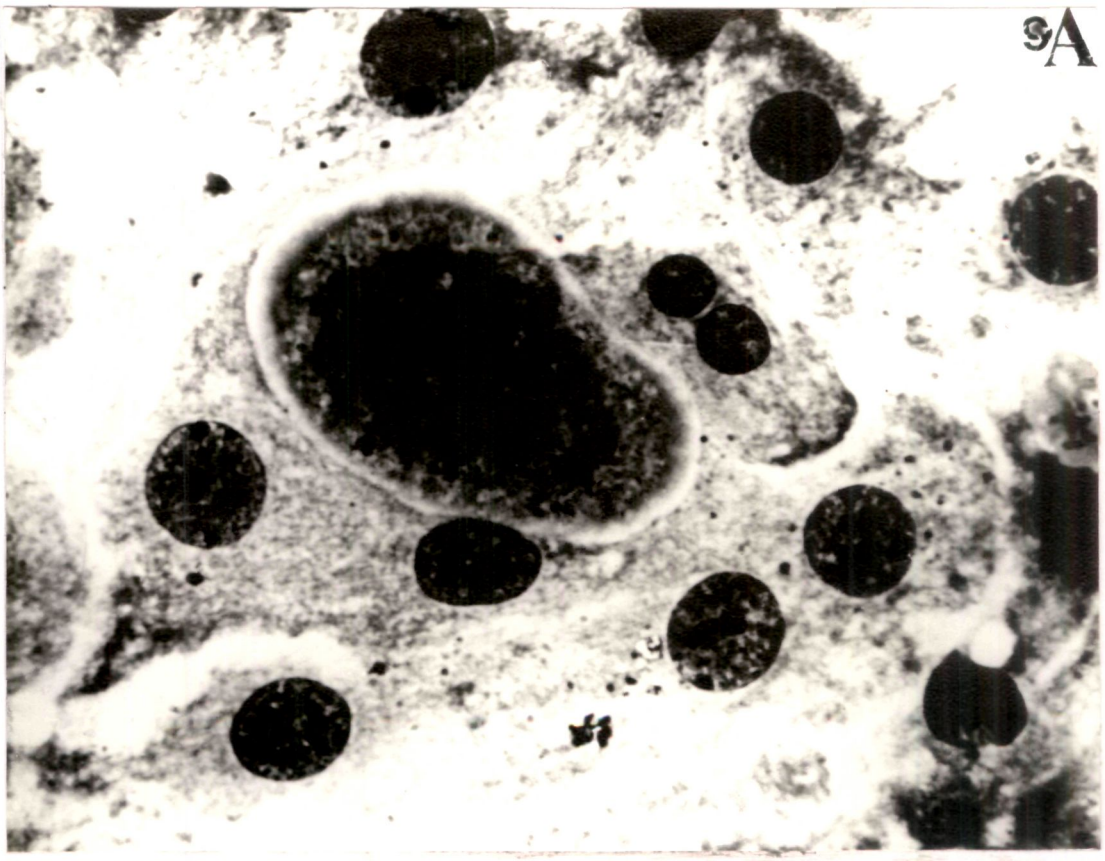

Cytochrome P-450 Content
(n moles/ mg Protein)
0·90
0·85
0·80
0·75
0·70
0·65
0·60
0·55
0·50
0·45
0·40
0·35
0·30
0·25
0·20
0·15
0·10
0·05
0
1
3
5
7
Days in Culture
Whole liver microsomes
Freshly isolated cells
Cells in synthetic medium
Cells in synthetic medium-Cyst(e)ine

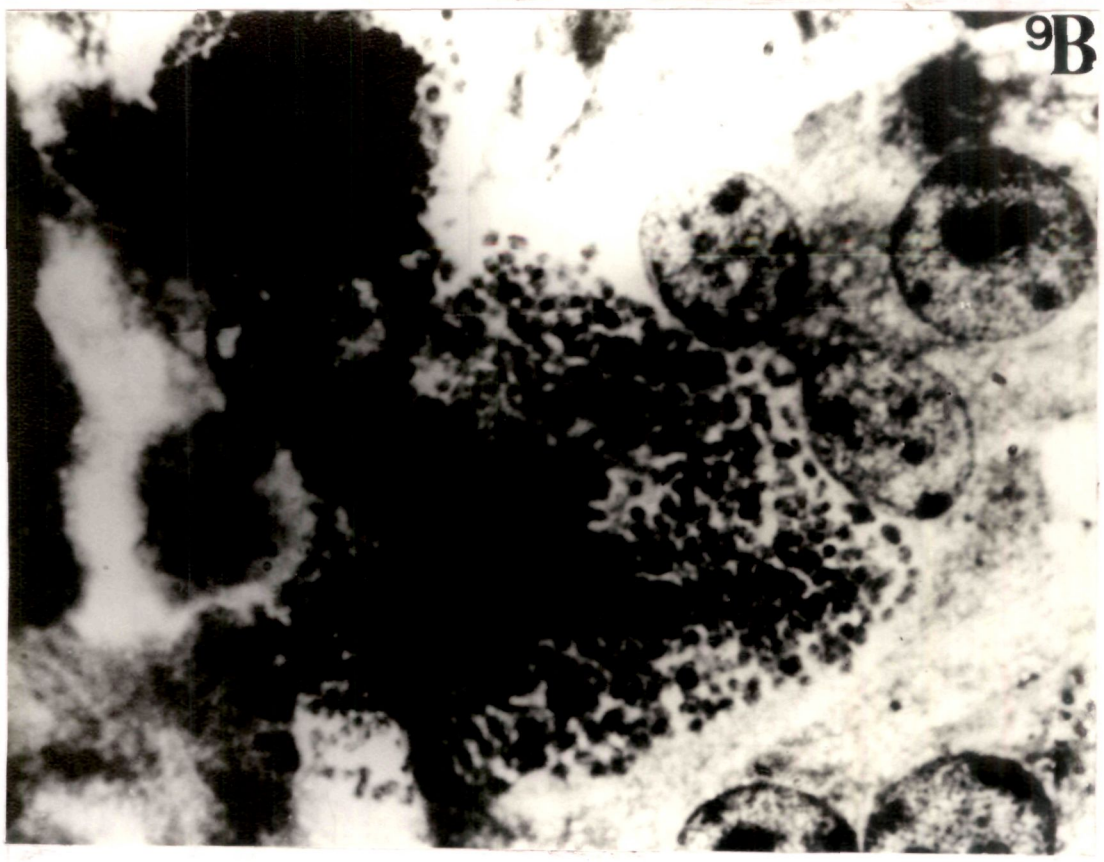
many developing EE forms. After 48 hrs, the schizonts ranged from 20 um to 40 um in diameter (Fig. 9A) and after 60 hrs, merozoites reported to be capable of invading and developing in reticulocytes in vitro were released from exo-erythrocytic schizonts into the medium (Fig. 9B). Although many sporozoites attached to and penetrated the hepatocytes, only a small fraction (0.1%) became schizonts.

Fig. 9: Rat hepatocytes infected with P. berghei sporozoites in vitro:

A) Giemsa stained schizont after 48 hr.

B) Exo-erythrocytic merozoites released into the medium after 60 hr.


9A


9B

D I S C U S S I O N

Primary culture of hepatocytes for long-term metabolic, pharmacologic or toxicologic investigations must fulfill atleast three fundamental criteria:

i) a substantial amount of the plated cells should survive the experimental period;

ii) non-liver specific functions must not increase;&

iii) liver-specific functions should be maintained.

A number of reports have documented extension of cell viability and short term modulation of several functions by corticosteroids in cultured hepatocyte (9, 89). However, although significant, these beneficial hormone effects were limited to a few days especially when parenchymal cells were incubated in serum-free medium. The findings described here demonstrate that hepatocytes expressing differentiated functions such as active albumin secretion, maintenance of cytochrome P-450 levels and O_2 uptake etc, might survive for several weeks when cultured in the presence of hormones and DMSO. When hepatocytes were cultured in DMSO supplemented medium, they remained viable for extended period. Because DMSO penetrates biological membranes — , the drug may function as a carrier for specific nutrients and hormones. DMSO also alters the structure of proteins and nucleic acids and as such may directly alter gene expression. The system described here uses hormonally defined synthetic medium and is ideal for testing whether DMSO acts by altering hepatocyte responsiveness to medium components.

Cytochrome P-450

The content of cytochrome P-450 decreases rapidly during cultivation of isolated rat hepatocytes. This decrease appears to be an adaptive response of cells grown under the culture conditions, rather than a reflection of declining viability of cells (17). The degradation process observed in the cultured hepatocytes resembles, in several respects, the physiological catabolism of cytochrome P-450 *in vivo*. In these cultured hepatocytes, the heme contained within cytochrome P-450 appeared to have been converted to bilirubin (20). This process is accompanied by increase in heme oxygenase activity (21). Lipid peroxidation has also been implicated in the degradation of cytochrome P-450 *in vitro* (79). Although numerous modifications of the culture medium have been formulated to attempt to retain the *in vivo* levels of cytochrome P-450, maintenance of cytochrome P-450 in cultured rat hepatocytes has been an elusive goal. Results of the current study show that the removal of cysteine from the medium and addition of DMSO supplemented synthetic medium protected the cells from the loss of cytochrome P-450 content for upto 7 days. After as long as 7 days in culture, levels of cytochrome P-450 in the cells grown in medium without cysteine were still comparable to cytochrome P-450 levels in freshly isolated hepatocytes.

Albumin synthesis and secretion

Adult rat hepatocytes continue to synthesize and secrete albumin *in vitro* (96). Synthesis rates are dependent upon the

protein tested, the species, the composition of the medium and the nature of the support. When the cells are cultured in standard conditions, a rapid decrease in albumin synthesis is observed. This decrease can be temporarily minimized by using a serum-free hormonally defined medium (42). Although not phenotypically stable in pure culture (18), adult hepatocytes retain a broad response to exogenous signals, particularly hormones and factors secreted, somewhat diminished, following culture shock (28). The study presented here showed that supplementation of medium with a non-physiological inducer, DMSO, not only enhanced albumin synthesis but also albumin secretion by the cells. Although it is possible to maintain-hepatocytes in differentiated state in a medium supplemented with hormones, growth factors and DMSO, further biochemical analysis is required to determine the normality of these hepatocytes with regard to other liver-specific functions, such as hormonal induction of specific enzymes, maintenance of cytochrome P-450 and ability to activate chemical carcinogens.

Hepatocyte cultures as screening model for hepatoprotective compounds

A number of xenobiotics either directly or after conversion to more toxic compounds, may impair hepatocellular and other functions. Cell injury may be initiated by the formation of a stable (non-covalent) complex with a protein or another intracellular compounds, or via the formation of highly reactive chemical species or by inducing physico-chemical changes within the cell or its

environment. Cell damage is limited or prevented by a variety of defence systems which include drug metabolizing enzymes, binding proteins, anti-oxidants and active oxygen metabolizing enzymes.

Cultured hepatocytes have been widely used as an experimental model for toxicity assessment (43). In the study presented here, cultured hepatocytes were used as screening model for evaluating toxicity of hepatotoxins. Cytotoxicity was evaluated by the measurement of cytosolic enzyme leakage, morophological changes visualized at the light microscopic level and determination of cell viability by the trypan blue exclusion test. Two cytotoxic agents, acetone and carbon tetrachloride were used to study their effects on the above parameters. The two caused an increased leakage of lactate dehydrogenase into the medium and reduced oxygen uptake by the cells. The hepatotoxicity of acetone has not been very well studied while that of CCl_4 can be attributed to the activation of the compound into highly reactive molecules (free radicals), by a mixed function oxidase process, which may occur either by cleavage by an electron transfer reaction or through interaction with free radicals. These extremely reactive electrophilic molecules with short half-lives bind rapidly and covalently to different liver macromolecular constituents thus causing a specific and predictable alterations in hepatocellular functions.

Several plant extracts and their isolates were found to be hepatoprotective in arresting Ac_2O induced impairment of plasma membrane leading to leakage of LDH and inhibition of O_2 uptake

by the hepatocytes. Some of them are quite promising (e.g. TR/17, TR/37, TR/77, TR/78 etc.). Needless to say, this is only a preliminary screen and it would be pertinent to follow them up in detail. Further, these screens only indicate inhibition of toxic manifestations due to acetone. However, it would be pertinent to look at effect of the compounds on sequalae to xenobiotic toxicity, particularly arresting the pathway leading to hepatic fibrosis or cirrhosis.

Effects of DMSO and Se

DMSO when added concurrently prevented the cytotoxicity due to CCl_4 and Ac_2O. It is premature to explain the exact mechanism underlying such a protection. But plausible explanations could be sequestration of the active electrophilic species, thereby rendering them ineffective. Further DMSO could also act as an oxygen radical sink. In addition, other reactions of DMSO involving methylation of intracellular macromolecules cannot be excluded. Se has proven antioxidant properties and so the protection afforded by its addition to medium is suggestive of role of antioxidants in hepatoprotective function.

Exo-erythrocytic stage cultivation *in vitro*

In vitro cultured hepatocytes have a potential in understanding host parasite interaction, particularly in the parasites which undergo pre-erythrocytic stage such as malaria. Further, the relapse in malaria as observed in *P.vivax* infection (87), necessitates develop-

ment of in vitro model for understanding molecular events in differentiation of sporozoite during exoerythrocytic schizogony as well as dormancy during hepnozoite stage. Recent report concerning the role of acute phase proteins in arresting hepatic tissue shizogony of malarial parasite is intriguing. It may be a natural defence mechanism which may turn out to be of significance in understanding the mechanism underlying sporozoite entry and differentiation in hepatocytes. Besides the advances in recombinant DNA technology afford means to look for specific transacting factors for some genes crucial for rapid replication of the parasite during EE schizogony. In this regard enzymes of the pyrimidine synthesis seem to be natural targets of study. Some efforts are also going on in different laboratories to clone centromere of the rodent parasite. Such an approach is actually now needed to help design strategies for controlling the disease.

Results of the current study showed that the penetration rate of P. berghei sporozoites into cultured hepatocytes was very low (0.1%). The underlying reason for the low in vitro infectivity of sporozoites compared to their infectivity in vivo (31) is unknown. Considering the large number of sporozoites in each inoculum and the relatively modest number of exo-erythrocytic forms found, it appears that many of the sporozoites do not penetrate the cultured hepatocytes. It has been proposed that in vivo, sporozoites do not enter the hepatocytes directly but must first trans the Kupffer cells, where they may be processed in some way (146). Clearly

under the conditions used for these cultures, sporozoites enter at least certain hepatocytes directly and go on to develop into liver schizonts.

Consequent to success in hepatocyte culture, rapid progress has been reported in demonstration of *in vitro* culture of exo-erythrocytic stage of rodent as well as human malarial parasite. In fact, recently complete differentiation of sporozoite of *P.falciparum* into erythrocyte invading merozoite has been demonstrated (103).

Despite the success reported by laboratories abroad, almost no attempt has been made at the national level in this direction. It was, therefore, considered of interest to develop technology for the above so as to be in a position to use it for developing agents for arresting the onset of disease.

SUMMARY

Recent developments in cell culture technology have made possible longer survival times and better maintenance of differentiated functions of hepatocytes in primary cultures. In particular hepatotropic growth factors in combination with other soluble factors allow for the maintenance of normal adult hepatocytes in serum free medium. Rat hepatocytes were isolated by in situ perfusion technique described by Seglen and cultured in synthetic medium and DMSO. The maintenance of hepatocytes in primary culture was performed according to Enat et al. Extracellular matrices, collagen, poly-lysine and fibronectin were used as culture substrates to improve the attachment and survival of the cultured hepatocytes.

The hepatocytes, cultured during the present study, retained their differentiated characteristics for atleast one week. These cells were found to synthesize and secrete albumin at a rate comparable to freshly isolated hepatocytes. Similarly, use of defined medium helped in maintaining cytochrome P-450 activity for atleast one week.

A simple in vitro system was developed to study the effects of various hepatotoxic and hepatoprotective agents on the integrity of hepatocyte plasma membrane. Two cytotoxic agents Ac_2O and CCl_4 were tested for their ability to produce hepatotoxicity while DMSO and Se proved to be the best hepatoprotective agents. Primary cultures were also used as screening model for various natural plant products some of which were found to have significant hepatoprotective activity. Hepatoprotective activity was followed by evaluating

the degree of reversal of inhibition (induced by hepatotoxins) in terms of ability of the hepatoprotectives to prevent/arrest impairment of hepatocyte plasma membrane function (LDH leakage) and O_2 uptake using glucose as major energy source.

An *in vitro* EE stage for *P. berghei* could be demonstrated. However, the technique remains to be perfected because of problems of a very low reproducibility.

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